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Focus Review

Spatial organization of transmembrane receptor signalling

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The spatial organization of transmembrane receptors is a critical step in signal transduction and receptor trafficking in cells. Transmembrane receptors engage in lateral homotypic and heterotypic *cis*-interactions as well as intercellular *trans*-interactions that result in the formation of signalling foci for the initiation of different signalling networks. Several aspects of ligand-induced receptor clustering and association with signalling proteins are also influenced by the lipid composition of membranes. Thus, lipid microdomains have a function in tuning the activity of many transmembrane receptors by positively or negatively affecting receptor clustering and signal transduction. We review the current knowledge about the functions of clustering of transmembrane receptors and lipid–protein interactions important for the spatial organization of signalling at the membrane.

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Introduction

Receptor-mediated signalling is a highly complex, evolutionary conserved mechanism that allows communication between cells and their environment. Efficiency, high precision and specificity are required to transmit only relevant signals to the appropriate target cells. To ensure transmission of even weak signals, the receptor and their associated complexes can be modified by dynamic and reversible post-translational modifications, which in turn promote amplification and diversification of signalling in the cell (Deribe *et al*, 2010). An additional level of complexity comes from the organization of receptors in higher-order clusters. Receptors do not function as individual signalling units, but tend to associate in multimolecular complexes that can accommodate up to hundreds of molecules. Examples include the chemotaxis receptors in bacteria, the epidermal growth factor receptors (EGFRs) and T-cell receptors (TCRs) in mammalian cells (Kentner and Sourjik, 2006; Deribe *et al*, 2009; Manz and Groves, 2010). Receptors associate in such complexes through direct interactions, indirectly through adaptor molecules or through specific interactions with lipid microdomains (Simons and Toomre, 2000; Seet *et al*, 2006). The multimolecular signalling clusters may consist of homotypic or heterotypic receptor associations, and may also involve different molecules located in the membranes from adjacent cells.

Advantages of receptor clustering include restricted diffusion of receptors at the membrane and amplification of the signal as a result of simultaneous activation of multiple receptors. In this review, typical examples of receptors in signalling clusters will be described. The importance of these associations in regulating signalling specificity and sensitivity will be discussed, stressing that receptor co-operativity is absolutely necessary for the integration of multiple signals and for the achievement of a coordinated cellular response.

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Homotypic receptor clustering in *cis*

Many basic principles governing the organization and functional importance of receptor–receptor complexes come from the study of the largest subfamily of receptor tyrosine kinases (RTK), the Eph receptors (Ephs) and their ligands, the ephrins. Ephs and ephrins are mainly involved in the developmental processes and organ morphogenesis, regulating cellular processes such as cell attraction and repulsion, sorting and motility or cell survival and differentiation (Pasquale, 2005). The nine human isoforms of EphA receptors bind to five glycosylphosphatidylinositol (GPI)-linked ephrinA ligands, whereas five EphB receptors bind to three transmembrane ephrinB ligands. Ligand–receptor specificity is low within the classes and even some inter-class associations have also been shown for ephrinA5 binding to EphB2 and the three ephrinB ligands binding to EphA4 (Pasquale, 2005, 2008). Upon interaction of Ephs with their ephrin ligands, signalling cascades are initiated in both cells carrying either receptor or ligand resulting in a bidirectional mode of signalling (see below under section 'Receptor associations in *trans*'). Once receptors and ligands from opposing cells come into contact, bidirectional downstream signalling occurs only after tetramerization of the *trans*-complex (Pasquale, 2005).

Initially, pre-clustered ephrins form homooligomers, which bind the Ephs with a 1:1 stoichiometry upon cell–cell contact. Further clustering progresses by the assembly of Eph–ephrin dimers into tetrameric complexes, inducing conformational changes on both the receptor and the ligand (Himanen and Nikolov, 2003). The Eph tyrosine kinase domains can then *trans*-phosphorylate each other and promote forward signalling, whereas recruitment of Src-family kinases (SFK) to ephrinA/B ligands and phosphorylation of the ephrinBs initiate reverse signalling. In a unique way, uncommon for RTKs, the tetramers can be further clustered in higher-order assemblies regulating the mode and strength of signalling.

Even though the binding of Ephs to pre-clustered ephrins is of catalytic importance for multimeric clustering and signalling efficiency, it seems that Ephs also have the ability to associate in homotypic complexes through their extracellular (Himanen *et al*, 2010; Seiradake *et al*, 2010) or/and cytoplasmic (Lackmann *et al*, 1998; Wimmer-Kleikamp *et al*, 2004) domains. At low receptor concentration, pre-clustered ephrin ligands are required for initial receptor clustering. However, above a certain concentration threshold, free EphAs can cluster through interactions of their ectodomains, independently of ligand binding. Taking advantage of this mechanism, even minimal amounts of ephrins can function as 'nucleation seeds' and can cluster a small number of Ephs, which can then initiate the recruitment of more receptors at the area of initial cell–cell contact and strengthen intercellular communication. In such a way, by co-operative hetero- and homomeric interactions, even weak signals can be amplified and coordinated to achieve efficient signalling (Himanen *et al*, 2010; Seiradake *et al*, 2010). Clustering of receptors that have an intrinsic enzymatic activity (such as the Ephs) is a direct mechanism to enhance downstream signalling. Nevertheless, receptor clustering seems to have additional functions. A constitutively active form of EphA4 that is phosphorylated without the need of ligand binding and stimulation, fails to regulate several developmental processes unless it becomes clustered by ephrinB (Egea *et al*, 2005). The reason for this might be the necessity of accumulation of downstream signalling effectors. A similar mechanism seems to be used by the ligand ephrin, which does not possess any kinase activity. It has been shown that ephrinBs require SFKs for their phosphorylation upon receptor engagement (Palmer *et al*, 2002). Activation of SFKs is achieved by auto-*trans*-phosphorylation. EphrinB clustering upon Eph-stimulation increases the local concentration of ephrin-recruited SFKs at the signalling foci, ensuring efficient SFK auto-activation and downstream signalling (Palmer *et al*, 2002).

Importantly, size and spatial patterning of signalling assemblies are not of random importance. On the contrary, these factors significantly contribute to the specificity of the signalling outcome, with small variations often resulting in opposite cellular responses. It is well known that only clustered or membrane-presented (and not free-soluble) ephrinB1 can induce phosphorylation of EphB1 (Davis *et al*, 1994). Moreover, different multimeric states of the Eph–ephrin complex (dimers, tetramers or higher-order multimers) result in distinct cellular responses. Even though dimeric-ephrinB1 can induce EphB1 phosphorylation, higher multimeric states of receptor complexes are necessary for the recruitment of downstream effectors and promotion of cell attachment (Stein *et al*, 1998). In accordance with this, EphB1 receptors have been successfully characterized as 'ligand density sensors' that modulate integrin-mediated cell–matrix attachment according to the density of the ephrinB1 that they encounter (Huynh-Do *et al*, 1999). Even though the Ephs are phosphorylated after exposure to low-density ephrinB1 ligands, they are only able to induce $\alpha v\beta 3$ integrin-driven cell attachment within a certain higher range of ephrinB1 density presented. Variations in the ephrinB1 concentration above or below this threshold result in the complete opposite phenotype, decreasing cell attachment. Application of the same principle could easily explain other examples of bimodal function of ephrinB1 acting as an attractant or repellant in retinal ganglion cell branching (McLaughlin *et al*, 2003) or of EphA–ephrinA signalling in promoting or inhibiting axonal growth (Hansen *et al*, 2004).

Further understanding of the mechanisms of multimeric receptor assembly was recently achieved by studies in which the size and shape of receptor complexes were not determined by the availability of the ligand, but was forced to desired configurations by mechanical artificial barriers ('spatial mutation approach') (Groves, 2006). In an elegant study, Salaita *et al* (2010) confirmed the already described models and provided a direct insight on how the clustering pattern of Ephs at the cell surface is reflected on intracellular actin arrangements (Figure 1A). They geometrically constrained the distribution of ephrinA1 on synthetically engineered membranes and accordingly, modified the membrane patterning of EphA2 in a living cell. As expected, the mobility of ephrinA1 on the supported membranes defined to a great degree the transport and function of EphA2 receptor at the plasma membrane. Inability of ephrinA1 to freely diffuse and form microclusters had a direct impact on EphA2 forward signalling by decreasing the phosphorylation and degradation of the receptor. Interestingly, interference with the size and pattern of clusters formed by EphA2 did not affect the phosphorylation status of the receptor, but it had a strong impact on the intracellular distribution of different downstream effectors such as f-actin, as well as the amount of recruited ADAM2 to the EphA2–ephrinA1 clusters (Figure 1A). Moreover, applying the spatial mutation on a library of breast cancer cell lines revealed a strong correlation between the radial membrane transport of EphA2 and the invasiveness of the cells.

Interestingly, these results are highly reminiscent of how the differential spatial patterning of TCRs elicits different signalling outcomes at the immunological synapse. The building of the highly organized multimolecular structure of the immunological synapse is initiated by the recognition of antigen-presenting cells (APCs) from T cells. TCRs recognize and bind major histocompatibility complexes (MHCs) that are presenting specific antigenic peptides (Fooksman *et al*, 2010). The activated MHC–TCR complexes are then transferred with the help of the actin cytoskeleton to the centre of the synapse, in which hundreds of molecules can accumulate. At the same time, intercellular adhesion molecule complexes organize at the periphery of the synapse, broadening the contact area between the T cell and the APC (Manz and Groves, 2010).

The organization pattern of the TCRs in the synapse tightly regulates the signalling potential of the receptors, but at the same time the stimulus strength seems to determine the signalling outcome of the receptors clustered at the centre

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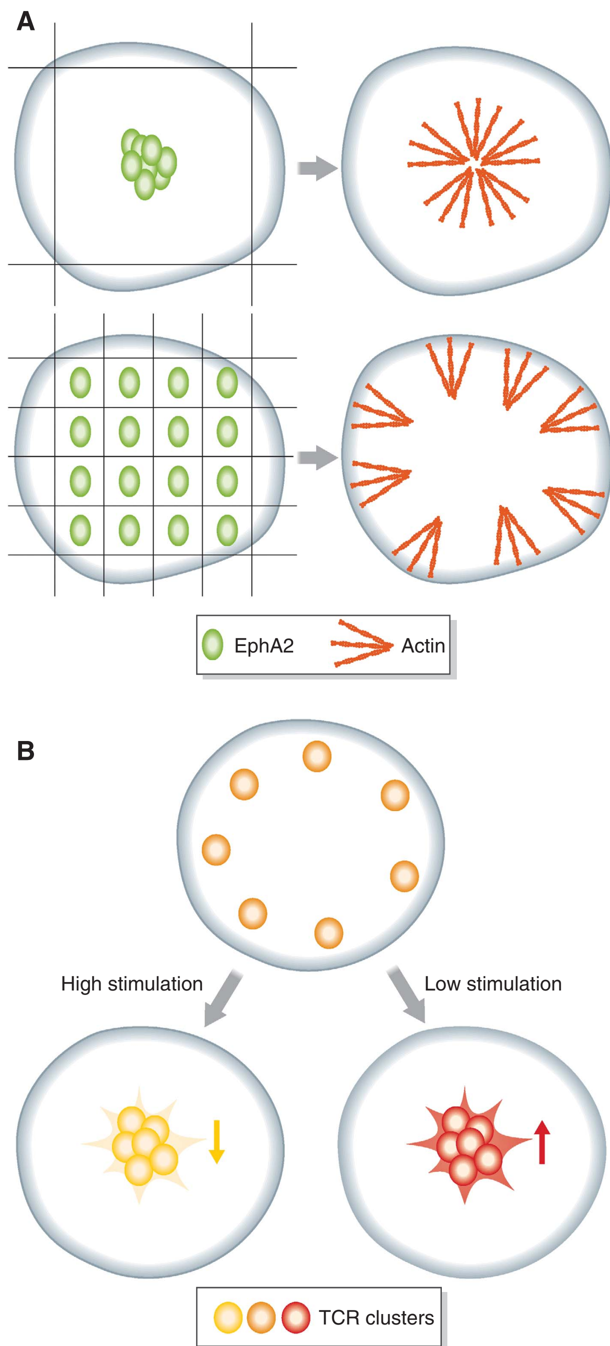

Figure 1 Spatial distribution of membrane receptors. (**A**) The size and organization of EphA2 membrane clusters determines the distribution of downstream effectors. Under conditions of unrestricted membrane transport of EphA2 receptor (green), f-actin (red) accumulates in the periphery of activated ephrinA1–EphA2 clusters. Upon introduction of a spatial mutation that restricts EphA2 organization at the membrane, the distribution of f-actin shifted to the cell periphery (Salaita *et al*, 2010). (**B**) Re-distribution of T-cell receptors (TCRs) to the centre of the immunological synapse results in different signalling states depending on the stimulus strength. Upon high stimulation, transport of TCRs (yellow) to the centre of the synapse results in receptor deactivation and attenuation of signalling (pale yellow receptors). In contrast, under low-stimulation, the artificially-forced translocation of TCRs to the centre of the synapse enhances downstream signalling.

of the synapse. Under high-stimulation conditions, the transport of TCRs to the centre of the immunological synapse leads to an attenuation of the signalling by receptor dephosphorylation, inactivation and endocytosis (Lee *et al*, 2003) (Figure 1B). Blocking this translocation step by artificial barriers ('spatial mutation approach') prolongs the presence of the receptors at the periphery of the synapse and results in a stronger T-cell response (Mossman *et al*, 2005) (Figure 1B). On the contrary, under low stimulation, the translocation of TCRs to the centre of the synapse helps enhancing the receptor signalling. Indeed, when the receptors are experimentally forced to occupy the centre of the synapse under conditions of low stimulation, the T-cell response is strongly enhanced (Cemerski *et al*, 2008) (Figure 1B). This example highlights the dual interplay between signalling regulation and receptor spatial organization: specific membrane arrangement of TCRs upon stimulation is essential for the efficiency of signalling, but at the same time the intensity of the signal seems to dictate how the positioning of the receptors in the synapse is perceived and 'translated' by downstream signalling, such as phosphatases or the endocytic machinery.

Receptor clustering is not only determining signal specificity, but can also contribute to the increase in cellular sensitivity to external stimuli or can even have a purely mechanistic function, by enhancing the strength of cellular contacts to the extracellular matrix. For example, signalling sensitivity highly depends on the organization of receptor complexes on bacterial membranes upon chemotaxis. These macromolecular membrane associations consist of the chemotaxis receptors, adaptor molecules and downstream kinases. Interactions between the chemoreceptors are the critical determinant of cluster organization (Kentner and Sourjik, 2006). Thousands of molecules can accumulate in distinct signalling clusters, in a nucleation process possibly using trimers of receptor dimers as basic building blocks (Ames *et al*, 2002; Li and Hazelbauer, 2004). Following a yet-unclear mechanism, chemoreceptor clusters show an additional level of spatial organization, by preferentially localizing at the poles of the cell. Nevertheless, the important feature of these clusters is that receptors of different types co-exist in these associations and functionally interact in a highly orchestrated manner (Gestwicki and Kiessling, 2002; Sourjik, 2004; Studdert and Parkinson, 2004). This organization results in the formation of allosteric multimolecular complexes that function as one signalling network; multiple signals are perceived by the same complex because of its diverse content in receptor types. Conformational changes of stimulated receptors increase allosterically the sensitivity of other receptors for their ligands and, therefore, efficient signal transduction and amplification of weak signals are ensured (Sourjik, 2004; Sourjik and Berg, 2004). Another example of signalling enhancement by receptor clustering can be found on the integrin signalling system. Integrins are a family of α/β heterodimeric cell-surface receptors and the major mediator of cell attachment to the extracellular matrix. They are able to signal across the membrane in both directions and are often found in highly organized clusters on the cell surface (Arnaout *et al*, 2005). Receptor association seems to be mediated by ligand binding and is important for their function. But some integrins can form clusters independent of ligand stimulation (Li *et al*, 2004), possibly through association of their transmembrane domains (Li *et al*, 2003). Furthermore, it was shown that extensive receptor clustering enhances cell adhesion by increasing the contact area

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between the cell and the matrix and, therefore, the strength of binding (Hato *et al*, 1998; Chen and Moy, 2000). Cross-linked receptors bind the substrate in a co-operative manner and can resist more efficiently to detachment forces. In contrast, under a random distribution of individual receptors, the same forces would be unevenly exerted in fewer and weaker connections, increasing the risk of breaking. The application of modern microscopy and biophysical techniques has now provided mechanistic details on how weak associations mediated by single integrin molecules turn into strong adhesive cellular forces by receptor co-clustering and co-operativity (Gallant and García, 2007; Taubenberger *et al*, 2007).

Lipid microdomains in receptor organization and signalling

Ligand-induced receptor clustering in the membranes is also influenced by the lipid composition of membranes and a network of protein–lipid interactions. The theoretical number of cellular lipids is close to 200 000 species, including $>$100 000 glycosphingolipids, 9600 phospholipids, almost 70 000 mono-, di- and triglyceride variants, as well as numerous fatty-acid and sterol-based varieties (Yetukuri *et al*, 2008). This astounding number may give the impression of a crowded and randomly packed membrane, with little room for joint behaviour between lipids. However, the exact opposite is true. The lipids are meticulously organized when it comes to localization and concentration in the plasma membrane versus organellar membranes, composition in outer versus inner membrane bilayer, as well as in its planar distribution within the membrane leaflets (van Meer, 2005). These differences determine the functionality of the lipids.

Although the idea of an ordered membrane structure received attention already in the 1970s (Jain and White, 1977), the concept of membrane microdomains or so-called lipid rafts saw light when it was first shown that GPI-anchored proteins and glycosphingolipids are enriched in detergent-insoluble membrane fractions (Brown and Rose, 1992; Simons and Ikonen, 1997). Lipid rafts are now defined as microdomains in the plasma membrane enriched in cholesterol, sphingolipids and certain proteins. For the past couple of decades, however, it has been questioned whether lipid rafts are fact or artefact (Munro, 2003). It is widely accepted that the method commonly applied to determine whether a protein is lipid raft associated, detergent extraction, lacks a solid physical basis. At the same time, little or no consideration has been taken for the artefacts that can be induced by this method. One clear example of the effect of detergent extraction on the cell membrane was shown by microscopy, showing that the plasma membrane remained predominantly intact with a few large 'holes' (Hao *et al*, 2001). This clearly does not correspond to the idea that detergent treatment solubilizes the membrane, leaving smaller microdomains intact. Another point that has caused debate is that the distribution of GPI-anchored raft proteins generally appears even across the plasma membrane when visualized by light microscopy, not in clustered units as expected (Mayor *et al*, 1994). Such observations have led to the speculation that the outer leaflet of the plasma membrane is 'one big raft' with small regions of fluid lipids in between. However, by moving away from detergent extraction, the existence of nanoscale assemblies of raft proteins in the membrane has now been shown by different techniques (Lingwood and Simons, 2010). Single-particle tracking (Suzuki *et al*, 2007), fluorescence correlation spectroscopy (Lenne *et al*, 2006), high spatial and temporal-resolution fluorescence resonance energy transfer (Goswami *et al*, 2008) and high-resolution imaging (STED microscopy) (Eggeling *et al*, 2009) in living cells show temporal nanoclusters of GPI-anchored receptors.

As an attempt to fit old and new data in one lipid raft model, a revision of the classical view of lipid rafts as pre-existing structures in the plasma membrane (Simons and Ikonen, 1997) has been proposed (Hancock, 2006). The revised model proposes that small, unstable liquid-ordered domains are formed spontaneously. Larger, more stable domains can then form when proteins are recruited. The upper size of rafts is limited because larger rafts will be captured by endocytosis, disassembled in the endocytic pathway and returned to the plasma membrane as separate lipid and protein components (Hancock, 2006). Importantly, this model proposes that proteins have an active function in raft formation. The requirement for protein–protein interactions in surface distribution has been elegantly shown by two independent studies showing that the minimal lipid anchor of lymphocyte-specific protein tyrosine kinase (Lck) or Lyn fused to GFP, respectively, was insufficient for microdomain localization as seen with the full-length proteins (Douglass and Vale, 2005; Larson *et al*, 2005). Overall, recent work supports the lipid raft model, and the focus is now being shifted towards understanding the function of lipid rafts in certain processes rather than questioning their existence.

Lipid rafts have a function in various cellular processes, including endocytosis and signal transduction (Simons and Toomre, 2000; Lajoie and Nabi, 2007). It has been suggested that lipid rafts may serve as signalling 'hot spots' largely based on the observation that many known signalling molecules are enriched within them (Simons and Toomre, 2000; Foster *et al*, 2003). One critical constituent of lipid rafts is cholesterol. An important structural feature of this lipid is its asymmetrical geometry resulting in two distinct faces, a smooth α-face and a rough β-face. This quality allows cholesterol to interact with two discrete membrane molecules simultaneously; for example a sphingolipid through the α-face and a transmembrane protein through the β-face (Fantini and Barrantes, 2009). Sphingolipids can additionally interact with the receptor and affect its conformation, showing a tight regulation between transmembrane receptors and their lipid environment.

One family of transmembrane receptors associated with lipid rafts constitute the G-protein-coupled receptors (GPCRs). The mechanism for raft association is still not clear for all the GPCRs, as there is no one general sorting signal. For some of the receptors, however, the activity status and the raft localization seem to be coupled. For example, the affinity state of human oxytocin receptor depends on structural features in cholesterol (Gimpl *et al*, 2000), suggesting that cholesterol interaction regulates receptor activation. Similarly, it is mainly the active version of the δ-opioid receptor that is found enriched in lipid rafts (Alves *et al*, 2005) (Figure 2A). A possible explanation for this could be the conformational change induced by activation, leading to a longer version of the receptor with a larger hydrophobic region. According to the hydrophobic matching hypothesis (Jensen and Mouritsen, 2004), this version of the receptor

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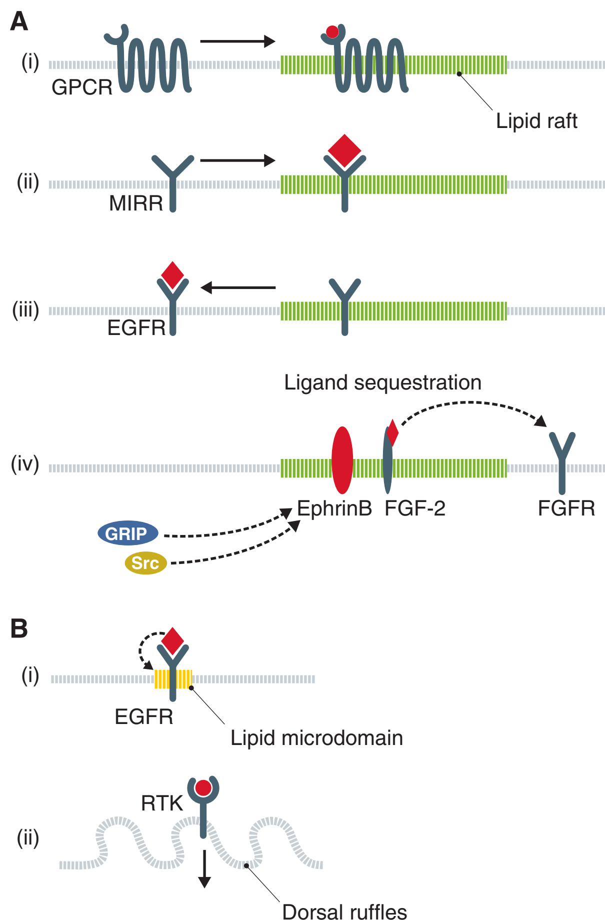

Figure 2 Lipid microdomains in receptor organization and signalling. (**A**) Lipid rafts (green) can serve to recruit ligand (red) stimulated receptors, such as (i) G-protein-coupled receptors (GPCRs) or (ii) multichain immune recognition receptors (MIRRs) and thus focus the downstream signalling complexes. (iii) The receptor tyrosine kinase (RTK) epidermal growth factor receptor (EGFR) is localized to rafts in resting cells and is transported out of the raft upon ligand binding. (iv) A third function for rafts in signal transduction is to sequester receptor ligands. EphrinB is a transmembrane ligand associated with rafts, and serves to recruit cytoplasmic effector molecules to this domain. The FGF-2 ligand is kept in rafts by glypican-1 and away from the non-raft-associated fibroblast growth factor receptor (FGFR). (**B**) Other microdomains in the membrane can also serve to spatially arrange transmembrane receptors. (i) Upon ligand stimulation, the EGFR induces the formation of a local lipid domain rich in phosphatidic acid (yellow), which also contains an elevated number of EGFRs. (ii) Dorsal ruffles can be induced by stimulation of RTKs. These domains may serve both as signalling platforms and as internalization sites.

will be redistributed into the thicker sphingomyelin-enriched bilayer. Other proposed mechanisms for raft localization of GPCRs include modifications by fatty acids and interaction with caveolin, a protein found in a subpopulation of lipid rafts, caveolea (Chini and Parenti, 2004).

RTKs make up a second family of transmembrane receptors associated with lipid rafts. The EGFR shuttles in and out of lipid rafts, and it has been shown that interactions between the extracellular receptor region and the GM1 ganglioside participate in targeting the protein to rafts (Miljan *et al*, 2002). Interestingly, in contrast to the GPCRs mentioned above, it is the inactive form of EGFR that is associated with lipid rafts, and the receptor moves out of the raft in response to EGF (Mineo *et al*, 1999; Roepstorff *et al*, 2002) (Figure 2A). EphrinB ligands are also localized within rafts, inducing the recruitment of a downstream signalling complex to these domains (Brückner *et al*, 1999; Palmer *et al*, 2002) (Figure 2A). An interesting twist to the regulation of fibroblast growth factor receptor (FGFR) signalling was recently reported. The receptor is localized in non-raft membrane in both its active and inactive state, whereas the ligand FGF-2 is sequestered in lipid rafts by the heparin sulphate proteoglycan glypican-1, preventing receptor binding and thus allowing skeletal muscle differentiation (Gutiérrez and Brandan, 2010) (Figure 2A).

Rafts appear to have an important function in immune cell activation. Cells of the innate and adaptive immune systems express multichain immune recognition receptors (MIRRs), which respond to the presence of foreign macromolecules. These receptors include TCRs, B-cell receptors and certain receptors for the Fc regions of antibodies. In resting cells, MIRRs show no or very limited association with rafts. Upon ligand stimulation and receptor oligomerization, however, the receptors translocate into rafts (Cherukuri *et al*, 2001) (Figure 2A). Similar to certain GPCRs, it is likely that the recruitment is due to a conformational change in the receptor, which results in a high affinity for the raft environment. In the rafts, the receptors associate with SFKs and initiate downstream signalling events.

In membranes, receptor activation may also induce changes in the lipid microenvironment. The EGFR is a single transmembrane protein organized into oligomers at the plasma membrane in a cholesterol-dependent manner. Upon ligand binding, the tyrosine kinase domain is activated and induces downstream effects including hydrolysis of plasma membrane phosphatidylcholine by phospholipase D2 to produce phosphatidic acid (PA) and choline (Ariotti *et al*, 2010). This creates a local microdomain rich in PA, which also contains an elevated number of EGFRs (Figure 2B). As the EGFR can bind to acidic lipids in the plasma membrane, it is likely that the receptor interacts with newly synthesized PA to form this protein–lipid complex (Ariotti *et al*, 2010).

Stimulation of RTKs with platelet-derived growth factor, epidermal growth factor or hepatocyte growth factor (HGF) induces dorsal ruffles or 'waves' in the plasma membrane (Mellström *et al*, 1988; Dowrick *et al*, 1993; Shinohara *et al*, 2002; Orth *et al*, 2006). Activated EGFR and Met RTK are recruited into these ruffles and internalized (Orth *et al*, 2006; Abella *et al*, 2010) (Figure 2B). Active Met shows a prolonged localization to dorsal ruffles, suggesting that these structures may serve as signalling platforms. Interestingly, internalization of Met from the dorsal ruffles also enhances their initial, but not total, degradation (Abella *et al*, 2010). The ruffles may, therefore, serve to focus downstream signalling, but at the same time more efficiently 'turn off' the signal.

Heterotypic receptor clustering

Efficiency in signalling and most important in specificity is not only achieved by the membrane microdomains and homotypic clustering of receptors, but is also enhanced by the co-operative spatial accumulation of different receptor types. The co-existence of different receptors in the same signalling complex determines the molecular and cellular context that each receptor is facing and modulates accordingly its signalling outcome. Co-operative receptors can have

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agonist or antagonistic functions, activate each other without the need of their ligands, change each others ligand sensitivity (see above chemotaxis receptor clustering) or can simply influence each others membrane targeting and trafficking. Merging signalling pathways by receptor cross-talk can serve as a mechanism to efficiently integrate multiple environmental signals to one common signalling pathway, enabling direct interpretation of external stimuli by the cellular machineries.

Known for their function as guidance cues during development, semaphorins are a group of receptors that often require multiple co-receptors to exhibit their function. Semaphorins associate mainly with plexins, but their signalling might require their interaction with neuropilins (Npns) or the Ig superfamily cell adhesion molecules (IgCAMs). These different associations further determine the outcome of the semaphorin signalling (Zhou *et al*, 2008). For instance, Sema3s do not interact directly with plexinA receptors, but first bind to Npn-1 or Npn-2 to get incorporated in Npn–plexinA holoreceptor complexes and induce signal transduction through the plexins. Whether the outcome of this signalling event will lead to repulsive or attractive axonal guidance actions further depends on the final recruitment of either IgCAM L1 or NrCAM to the multimeric receptor complex (Castellani *et al*, 2000; Falk *et al*, 2005). Sema 3E alone can bind plexinD1 and mediate endothelial or axonal cell repulsion. However, when Npn-1 joins the complex, Sema 3E signalling is translated into cell attraction (Chauvet *et al*, 2007). Similarly, Sema 7A regulates cell adhesion differentially by binding to different receptors–plexinC1 (Walzer *et al*, 2005) or to integrins (Scott *et al*, 2008). This necessity for heteromeric signalling complexes in regulating semaphorin function is not restricted to its known co-receptors, but also includes members from other signalling pathways. Sema4D binding to plexinB1 increases the kinase activity of the RTKs Met and Erb2, mediating cell migration and metastasis (Giordano *et al*, 2002; Conrotto *et al*, 2005) or inducing cell migration and growth-cone collapse (Swiercz *et al*, 2004, 2008), respectively. Similarly, plexinA1 needs to form holoreceptor complexes with VEGFR2 to promote Sema6D-mediated signalling during cardiac development (Toyofuku *et al*, 2004).

Following similar principles, it has been realized in the recent years that Ephs and ephrins also cross-talk physically with other receptors to mediate many of their biological functions (Figure 3). For example, Ephs have been heavily implicated in cancer development and progression and their positive or negative contribution to these events highly depends on their functional association with known oncogenic growth factor receptors. Thus, EphA receptors can function as tumour suppressors when they are solely activated by their ephrin ligands (Pasquale, 2010), but can turn into potent tumour enhancers, through their associations with oncogenic receptors, such as the members of the EGF receptor family. In a transgenic mouse breast cancer model, Erb2 was shown to physically and functionally interact with EphA2, inducing EphA2 phosphorylation in the absence of ephrin ligands (Brantley-Sieders *et al*, 2008). Most importantly, the EphA2–Erb2 association was required for the maximal activation of the Erb2-downstream signalling cascade and seemed to account for the observed tumour proliferation and metastatic potential in Erb2-overexpressing mice. Conversely, in an earlier study, the activated EGFR and EGFRvIII (an oncogenic mutant form of EGFR) were shown to associate with EphA2, but this association led to a reduction of the EGF-induced cell migration (Larsen *et al*, 2007).

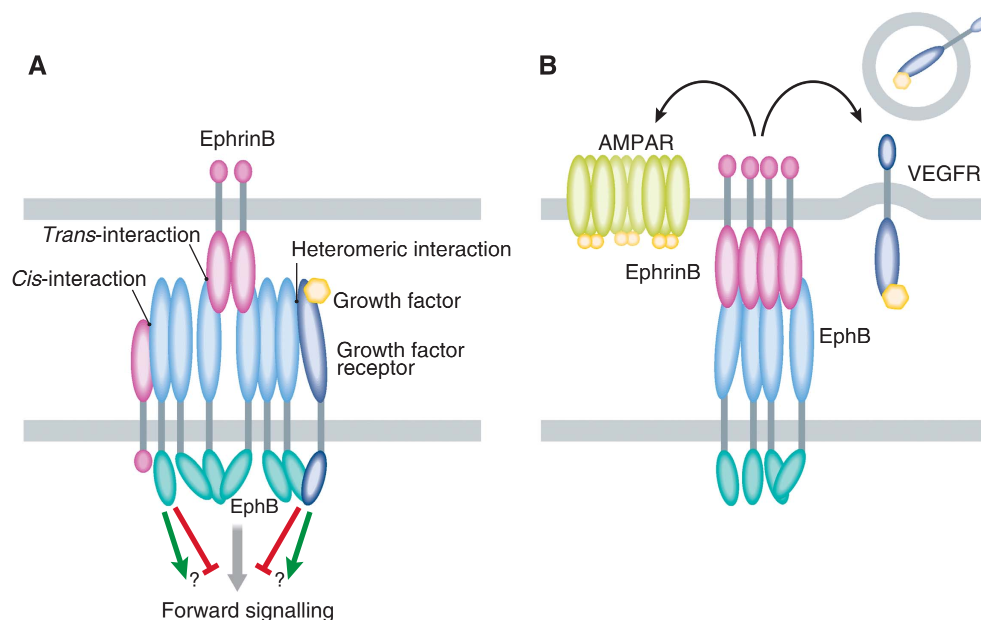

Figure 3 Receptor–receptor complexes regulate signalling specificity and receptor trafficking. (**A**) Eph receptors (Ephs) are clustered on the membrane through *trans*-interactions with pre-clustered ephrin ligands or through homomeric interactions of their extracellular or intracellular domains. Clustering is necessary for the receptor activation and signalling. The downstream signalling of activated Ephs can be further modulated by interactions with ephrin ligands in *cis* or by heteromeric associations with other receptor types. (**B**) EphrinB2 differentially regulates the trafficking of AMPA and VEGF receptors. Serine phosphorylation of ephrinB2 promotes AMPAR stabilization at the cellular membrane of neurons, whereas ephrinB2 positively regulates VEGFR endocytosis through its PDZ-binding domain.

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In glioma cells, the physical association of EphA4 and FGFR1 seems to be responsible for the observed enhancement of the FGFR-downstream signalling through the MAPK, Akt and Rac1/cdc42 pathways (Fukai *et al*, 2008). The increased cell proliferation and migration under conditions of EphA4 overexpression and FGF stimulation, does not seem to require ephrin ligands, as was shown for FGFR3 and EphA4 (Yokote *et al*, 2005). Owing to their physical interaction, the two receptors can *trans*-phosphorylate each other and activate the MAPK pathway. A similar principle of a functional cross-talk between EphA2 and the HGF-receptor c-Met could explain the activation of EphA2 and its positive impact on mammary epithelial proliferation and branching, upon HGF stimulation (Vaught *et al*, 2009).

Functional interactions between receptors that have antagonistic functions can provide an elegant fine-tuning mechanism of signalling regulation. Both ephrinB1 and the FGFR regulate retinal fate in Xenopus embryos. EphrinB1 promotes the retinal progenitor cell movement into the eye through its association with the scaffold protein Dishevelled (Dsh). The ability of FGFR to interact with ephrinB1 and induce its phosphorylation upon FGF stimulation disrupts the ephrinB1–Dsh interaction and results in suppression of the retinal fate (Chong *et al*, 2000; Lee *et al*, 2009). Similarly, activation of FGFR1 in EphB2-expressing cells interferes and blocks the ephrinB1-mediated cell repulsion and segregation, by increasing the steady-state phosphorylation of EphB2 and inhibiting the MAPK pathway in a feedback signalling cascade (Poliakov *et al*, 2008). At the immunological synapse, the presence of CD28 clusters surrounding the centred accumulation of TCRs promotes TCR signalling, but when CD28 molecules are experimentally forced to co-localize at the centre of the synapse with TCRs, cell signalling is decreased (Shen *et al*, 2008).

The formation of new synapses and the modulation of their activity primarily depend on the number of neurotransmitter receptors and their functional status. Modulation of synaptic morphogenesis and activity by the cross-talk of the Eph/ephrin bidirectional signalling with the NMDA and AMPA receptors constitute a perfect example on how receptor-coordinated function efficiently intermingles different signalling pathways in one developmental process. Mice lacking all three EphB isoforms expressed in the nervous system exhibit abnormal spine formation as well as low levels of NMDA and AMPA receptor clustering (Henkemeyer *et al*, 2003). Dalva *et al* (2000) showed that eprhinB1-mediated stimulation promotes the interaction of EphB2 with the NR1 subunit of the NMDA receptor and increases the clustering of NMDA receptors on dendritic membranes as well as the number of newly formed synapses on cultured neurons. Further evidence on the EphB2 and NMDAR co-operation comes from the facts that EphB2 can cluster NMDA receptors and promote spine formation (Contractor *et al*, 2002) and can enhance NMDA receptor phosphorylation (Takasu *et al*, 2002). Conversely, genetic depletion of EphB2 results in reduced NMDA-mediated currents and reduced synaptic localization of the NR1 subunit in neurons (Henderson *et al*, 2001). In addition, EphB2 co-clusters with AMPA receptors at synapses and regulates their activity, modulating in such a way spine development and mossy fibre long-term potentiation (Contractor *et al*, 2002; Kayser *et al*, 2006). But also reverse signalling by ephrinB ligands modulates the function of synaptic scaffold proteins or the trafficking of neurotransmitter receptors during synaptic plasticity and function. EphrinB2 modulates the trafficking of AMPA receptors—and, therefore, their activity—in a process that requires the bridging PDZ-containing protein Glutamate receptor-interacting protein1 and the serine phosphorylation of the ephrinB2 tail (Essmann *et al*, 2008) (Figure 3B). AMPA receptors become stabilized at the synaptic membrane by ephrinB ligands and lack of the ephrinB results in a constitutive internalization of AMPA receptors, which leads to impaired synaptic transmission (Essmann *et al*, 2008). Interestingly, recent work has revealed a novel cross-talk of ephrinB2 with the VEGF receptors. EphrinB2 associates physically with VEGFR2 (Sawamiphak *et al*, 2010) and VEGFR3 (Wang *et al*, 2010) at the membrane regulating the trafficking of these vascular receptors (Figure 3B). VEGFR2 and VEGFR3 endocytosis is required for the function of these receptors and ephrinB2 emerges now as a major controlling co-receptor needed to regulate the VEGFR signalling cascades during developmental and tumour angiogenesis (Sawamiphak *et al*, 2010) as well as lymphangiogenesis (Wang *et al*, 2010).

Receptor associations in *trans*

Receptor–receptor associations are not restricted in one membrane plane, but can adapt spatial configurations that serve intercellular communication. Receptors can be located on the membranes of different cells and associate in a *trans*-configuration, driving signalling cascades in both cells, in a process known as bidirectional signalling. The mode of receptor–receptor spatial organization in bidirectional signalling is imposed by the nature of their function as the receptors have to be spatially confined at the interfaces of cell–cell contact. However, receptor associations in *cis* are still possible and can interfere with the functions of the *trans*-complexes, so additional regulation on the spatial domain is required to ensure appropriate distributions between *cis*- and *trans*-assemblies. From the following examples, it becomes apparent that intercellular interactions among receptors involved in bidirectional signalling occur in a highly orchestrated manner and require mechanisms that discriminate them from identical receptor associations that take place in *cis*.

As previously discussed, Ephs and their ephrin ligands participate in intercellular signal transduction events. Activation of Ephs (forward signalling) results in auto-phosphorylation of their cytoplasmic kinase domain, further phosphorylation of downstream effectors and association with other protein adaptors, whereas ephrin activation (reverse signalling) also involves the phosphorylation of their cytoplasmic tails and interaction with multiple proteins. Eph and ephrin downstream signalling mediate cell proliferation, survival and differentiation, but mainly cell adhesion, shape and motility through cytoskeleton rearrangements. As these actions require the coordinate response of both signalling cells, it is not surprising that some of the molecules that are activated downstream of both receptor and ligand are common, such as the SFKs (Palmer *et al*, 2002; Knöll and Drescher, 2004) or the Rho-family GTPases (Noren and Pasquale, 2004). Nevertheless, a detailed proteomic and computational study revealed recently that the downstream signalling networks activated in the two participating cells

upon Eph/ephrin ligand binding are distinct, involving either different molecules or the same molecules, but regulated in opposite manners (Jørgensen *et al*, 2009). Cell repulsion and cell sorting is then achieved by this asymmetric and differential activation of downstream cascades in the two cell populations. Another interesting outcome from this work is the discrepancies observed in the cellular responses when the bidirectional signalling is transformed to unidirectional. Stimulation of EphB2-expressing cells with either soluble ephrinB1 molecules or membrane-bound ephrins that lack their cytoplasmic tail results in a different pattern of downstream signalling compared with the one induced by transmembrane wild-type ephrins. Removal of the cytoplasmic tail on the ephrinBs leads to an impairment of ligand endocytosis (Zimmer *et al*, 2003) that will thereby increase the concentration of ligands and will influence the balance and activation on the receptor side. This unbalanced situation leads to changes in the cellular behaviour outcome and can for example convert cellular repulsion into adhesion (Zimmer *et al*, 2003). Therefore, bidirectional signalling is not a cell-autonomous process—the functional bridge built by the interaction of receptors in *trans* regulates cellular responses depending on the molecular status of the co-signalling cell.

An interesting aspect concerning the spatial organization of receptor complexes involved in bidirectional signalling is the discrimination between *trans*- and *cis*-receptor associations and signalling. Both Ephs and ephrins have their own downstream pathways that can often induce complete opposite responses. For example, in neuronal axon targeting, EphA forward signalling results in growth-cone collapse and cell repulsion, whereas ephrinA signalling promotes axonal growth and attraction (Egea and Klein, 2007). An intriguing complication on how bidirectional signalling is regulated raises from the fact that both molecules are co-expressed on the growth cones of developing neurons. The work of Marquardt *et al* (2005) provided useful insight into this issue showing that both molecules manage to keep their signalling activities separate by segregating in distinct membrane microdomains thereby preventing their *cis*-association. The disruption of the membrane organization of co-expressed EphAs and ephrinAs using chimeric proteins results in massive *cis*-associations among receptors and ligands and a complete disturbance of the signalling patterns exhibited by the endogenous proteins. In another example of bidirectional signalling, the signalling outcome of Semaphorin3A can lead to cell attraction or repulsion depending on whether its interaction with the neuronal adhesion molecule L1-CAM and Neuropilin 1 occurs in *cis*- or *trans*-configuration (Castellani *et al*, 2002). Further complemented by additional studies that suggest the necessity of *cis*-interactions between EphA3 and ephrinA5 in regulating the developmental targeting of retinal axons (Hornberger *et al*, 1999; Carvalho *et al*, 2006), not only the absolute spatial distribution of receptor complexes, but also its relative positioning to other signalling clusters seem to be a necessary mechanism ensuring specificity in bidirectional signalling.

Another signalling pathway requiring direct cell–cell contact is the Notch signalling cascade. This evolutionary highly conserved pathway functions with an impressive degree of spatial and temporal specificity and it is of fundamental importance in a range of developmental processes, promoting cell differentiation and embryonic patterning. The members of the Notch signalling pathway are transmembrane proteins and consist of the Notch receptors and the members of the DSL-family Delta and Serrate/Jagged that act as ligands. In brief, the canonical signalling involves binding of Notch to its ligand, cleavage of Notch and the release of the Notch intracellular domain (NICD), in the cytosol. NICD is then translocated to the nucleus and regulates transcription of various developmental genes including these of Notch itself and its ligands, determining this way the fate of the activated cells (Bray, 2006). As with the previous examples, interactions between Notch and Delta ligands can occur both in *cis* and in *trans* leading to differential cellular outcomes. The Delta ligands have been shown to activate Notch when the proteins are located in opposing cells, but can inhibit its signalling when they interact in *cis* (Jacobsen *et al*, 1998; Miller *et al*, 2009). A recent study using advanced microscopy and modelling techniques shows that the coordinated action of *cis*- and *trans*-signalling might actually regulate the cellular fate determination mediated by Notch during development. Notch function specializes in amplifying small molecular differences between cells to promote differentiation. Sprinzak *et al* (2010) show that the strength of the Notch response to *trans*-Delta (activation) depends on the amount of presented ligand, but the response to *cis*-Delta (inhibition) occurs at a certain concentration threshold and does not depend on *trans*-Delta. They, therefore, suggest that on the multicellular level, this mechanism can be applied to read-out and subsequently amplify pre-existing, intercellular molecular differences and promote differential development of the interacting cells by creating boundaries and establishing lateral inhibition patterns.

Conclusions and future perspectives

Our understanding on signalling transduction mechanisms has been accelerated in the last decade. We have now a fairly broad knowledge about mechanisms regulating ligand–receptor interactions at the molecular and structural levels as well as the signalling cascades activated downstream of specific receptors. Studying the assembly and function of receptor complexes will advance significantly our understanding on receptor-mediated signalling and it is our next challenge to try to elucidate this extra level of complexity in receptor-mediated signal transduction. Which are the mechanisms that cluster or segregate receptors? What modes of receptor–receptor or receptor–lipid associations are conserved and important for receptor function? How can the activation of a single receptor be translated in different cellular responses depending on a differential organization and activation in space and time? Or vice versa, how can the cell coordinate the cross-talk among different receptors to achieve one single cellular response?

The study of this higher level of complexity achieved by the cross-talk of receptors or receptor–lipid interactions is of particular importance during the development of therapeutical strategies in disease. From this perspective, it is interesting to note that lipid rafts are more abundant in cancer cells than in normal cells, because of a generally elevated level of saturated fatty acids and cholesterol in these cells (Siddiqui *et al*, 2007). The increased level of raft lipids has been proposed to alter the lipid raft structure and consequently its protein composition (Rakheja *et al*, 2005). As major

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signalling proteins in large part are regulated by lipid rafts, one can easily imagine that such changes will have drastic effects on the cell. Indeed, it has been suggested that this may enhance cancer cell survival by promoting growth, escaping immune surveillance or preventing apoptosis (Rakheja *et al*, 2005). How to translate this knowledge into new therapeutics is still unresolved. It has been shown that cholesterol depletion of cancer cells renders them sensitive to apoptosis (Li *et al*, 2006). However, such an approach would be unspecific, and, therefore, limits its application in therapy. An alternative and promising strategy is to alter the lipid raft structure by controlling the uptake of dietary lipids (Siddiqui *et al*, 2007).

Other diseases such as obesity and insulin resistance also involve disordered lipid dynamics and membrane microdomains (Frühbeck *et al*, 2007). As briefly mentioned above, caveolea constitute a subgroup of lipid rafts, characterized by the presence of caveolin. The caveolin protein family is composed of three isoforms, caveolin-1, -2 and -3, with unique tissue distribution. Caveolin-1 knockout mice are lean and show insulin resistance and resistance to diet-induced obesity (Razani *et al*, 2002; Cohen *et al*, 2003). On the contrary, caveolin-3 knockout mice show increased body weight despite normal food intake, as well as insulin resistance (Capozza *et al*, 2005). These phenotypes may be relevant for human beings, as the human caveolin-1 gene is located in a chromosomal region associated with an obesity-related phenotype, and two mutations in the human insulin receptor associated with severe insulin resistance are located within the established caveolin-1-binding motif (Pérusse *et al*, 2005). Furthermore, a mutant mouse model unable to synthesize the GM3 ganglioside shows increased insulin sensitivity and is protected from fatty diet-induced insulin resistance (Yamashita *et al*, 2003). On the basis of this knowledge, novel therapeutic strategies are being proposed (Inokuchi, 2010).

Dysfunction in signal transduction pathways is often the main cause of diseases and cancer. The current therapeutic approaches target individual receptors and try to inhibit its ligand-mediated activation or the function of its downstream effectors. Such approaches seem ineffective when one considers the co-operative mode of action that most of the receptors exhibit in the cell. The signalling network built by clustering of multiple receptors complicates the manipulation of individual signalling pathways, as disrupting the signalling of one receptor type might trigger unpredictable reactions from other co-functioning signalling pathways leading, for example, to hyperactivation of redundant mechanisms that will result in a bypass of the particular targeted signalling pathway. Interfering with receptor clustering, either by preventing receptor–receptor associations or disrupting membrane lipid organization, might be an intelligent novel direction in receptor targeting.

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Conflict of interest

The authors declare that they have no conflict of interest.

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